THE EFFECTS OF SOME DEVELOPMENTAL INHIBITORS ON THE PHOSPHORUS BALANCE OF AMPHIBIAN GASTRULAE

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Considering the available evidence, it is possible that the morphogenetic movements of gastrulating amphibian embryos are energetically coupled to exergonic metabolic processes, and it is reasonable to assume that the coupling is mediated by energy-rich phosphate-bonds. On this basis, it is possible to propose explanations for the well-known inhibitory effects upon gastrular development of such agents as anaerobiosis, azide and dinitrophenol (Ornstein and Gregg, 1952; Gregg and Ornstein, 1953), for all of these are believed to dissever or restrict energetic couplings: anaerobiosis by switching out the aerobic exergonic processes of the Krebs cycle, azide perhaps by promoting the immediate remineralization of newly esterified phosphorus in the Embden-Meyerhof system (Spiegelman, Kamen and Sussman, 1948), and dinitrophenol perhaps by promoting the catalytic remineralization of esterified phosphorus by mitochondrial dephosphorylases (Hunter, 1951) or by direct "quenching" of energy-rich phosphate bonds (Middlebrook and Szent-Györgyi, 1955). If the energetic demands of gastrular movements are at all appreciable, then embryos treated with such agents might be expected to exhibit decreases in their stores of esterified phosphorus, accompanied by corresponding increases of their inorganic phosphorus contents. The experiments reported in the sequel are intended to test this proposal.

Methods

Obtaining and rearing embryos. Fertilized eggs were obtained by stripping eggs from gravid *Rana pipiens* females into suspensions of active sperm (*R. pipiens* or *R. sylvatica*). After about two hours, the clutches of embryos were cut with scissors into small groups, dispersed thinly among several finger bowls, and reared at a temperature of 14–15° C. until required for use. The medium in the bowls, changed daily, was 10% amphibian Ringer's solution without phosphate or bicarbonate. Just before use, the embryos were freed of their jelly-coats with forceps. Their developmental stages were determined by reference to the tables of Shumway (1940).

Treatment with inhibitors. Solutions of sodium azide and 2, 4 dinitrophenol were prepared by dissolving weighed samples in aliquots of the same medium in which embryos were reared. The treatment consisted in placing 25 or 30 Stage 10 embryos in a covered stender dish containing 5 or 10 ml. of inhibitor solution for 24 hours at 14–15° C. At the end of this period their developmental stages were noted, then they were washed rapidly with distilled water and lyophilized (see below). Afterward, they were dry-stored in the freezing compartment of a refrigerator until phosphorus analyses could be made, usually within two or three days.

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Anaerobiosis. For each experiment, 25 or 30 Stage 10 embryos were put into an Erlenmeyer flask fitted with a two-hole rubber stopper carrying a gas-inlet tube dipping into the medium in the flask (50 ml. of 10% amphibian Ringer's solution, without phosphate or bicarbonate) and a gas-outlet tube. Nitrogen alone, or 95% N_2 : 5% CO_2 (both previously deoxygenated over hot copper), or hydrogen, was then bubbled through the flasks for one hour, after which the inlet and outlet tubes were closed off with pinch clamps. Controls were prepared similarly, except that air, instead of nitrogen or hydrogen, was bubbled through the medium. After 24 hours (at 14–15° C.), the embryos were removed from the flasks, quickly staged, washed in distilled water and lyophilized. These steps preliminary to freeze-drying were carried out as rapidly as possible to prevent the occurrence of aerobic recovery processes. As before, frozen-dried embryos were stored for a short time, if necessary, in the freezing compartment of a refrigerator.

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Lyophilizing. Embryos were dried *in vacuo* in the frozen state with the help of an all-metal apparatus of conventional design built by Mr. Andrew Pfeiffer, Old Lyme, Connecticut. Washed embryos were placed with a minimum amount of distilled water in 10 × 75 mm. Pyrex test tubes which were then partly immersed in a Cellosolve-dry ice slush. After one or two minutes, the tubes were transferred to the drying apparatus. Drying was usually complete in three hours. Samples for chemical analysis were weighed out as rapidly as possible in a closed balance containing a silica-gel desiccant wafer, since lyophilized amphibian embryos are quite hygroscopic.

Phosphorus analyses. Total phosphorus (P_T), total acid-soluble organic phosphorus (P_{AO}) and inorganic phosphorus (P_I) were estimated, in micro- and semimicro amounts, using the methods of Lowry *et al.* (1954). Since we made no essential departures from their recommendations, the reader is referred to their paper for procedural details. The results of analyses are expressed as micrograms P per milligram dry weight of embryo.

Discussion of Results

Azide and dinitrophenol. At a temperature of 14–15° C., normal *R. pipiens* embryos will develop from Stage 10 (early gastrula) to Stage 12 (late gastrula) in about 24 hours. In the presence of inhibitors, morphogenesis may be reduced in amount or abolished altogether, with varying degrees of recovery following the cessation of inhibitory treatment (Table 1).

After 24 hours in 10^{-5} *M* azide, *R. pipiens* embryos immersed at Stage 10 will have reached early Stage 12, with no observable after-effects. Embryos similarly treated with 10^{-4} to 10^{-3} *M* azide will have gastrulated only partially, with after-effects ranging from slight to severe. In 10^{-2} *M* azide, no gastrulation occurs, and the after-effects are very severe.

After 24 hours in 10^{-7} *M* 2, 4 dinitrophenol, *R. pipiens* embryos immersed at Stage 10 exhibit no developmental peculiarities, and there are no detectable after-effects of this treatment. In 10^{-6} to 10^{-5} *M* dinitrophenol development is usually retarded in various degrees and the after-effects range from moderately severe to complete failure of recovery. Dinitrophenol in concentrations of 10^{-4} *M*, or greater, inhibits gastrulation altogether, and no recovery has yet been observed.

TABLE I

Development of R. pipiens embryos after treatment with azide or dinitrophenol, 14°–15° C. Stage 10 jelly-free embryos were immersed in inhibitors for 24 hours, then washed daily in 10% amphibian Ringer's solution until controls reached Stage 21. Sc_{12} = stage of treated embryos after 24 hours when controls are in Stage 12. Sc_{21} = stage of treated embryos when controls are in Stage 21

Inhibitor	Molar concentration	Sc_{12}	Sc_{21}	Morphological condition
Azide	10^{-5}	12^-	21	Normal
	10^{-4}	$10\frac{1}{2}$–12	20	Mostly normal. Gills underdeveloped but with circulation
	10^{-3}	10–11	18–20	Thickened tail buds, swellings on flanks, underdeveloped gills with circulation
	10^{-2}	10	18–19	Large yolk plugs, spina bifida, swellings on flanks, muscular response
2,4 Dinitrophenol	10^{-7}	12	21	Normal
	10^{-6}	11–12	11–18	Most in Stage 17–18, fairly normal, some with spina bifida
	10^{-5}	10^+–11^-	10^+–16	Most in Stage 10^+–11^-. Others misshapen, with large yolk plugs, whitish "bloom" on surface coat
	10^{-4}	10	10	"Bloom"
	10^{-3}	10	10	"Bloom"

Thus, it would seem that embryos which have been *totally* blocked with azide can afterwards attain a considerable degree of morphological maturity, something which is apparently denied embryos similarly blocked with dinitrophenol. Just possibly, however, this difference between azide and dinitrophenol may be less related to specific differences in their chemical activities than to differences in their separate abilities to pass outwards through the vitelline membrane. For, we found that even repeated washing fails to remove all of the dinitrophenol from treated embryos; part of it, at least, remains visibly concentrated in the perivitelline fluid. Whether there is a similar retention of azide we do not know, since solutions of this inhibitor are colorless.

We turn now to discuss the effects of such treatments upon the phosphorus balance of amphibian gastrulae.

TABLE II

Developmental stage and phosphorus balance of R. pipiens gastrulae after 24 hours exposure to sodium azide, 14–15° C. For meanings of column headings, see section on Methods. Numerals following ± designate standard deviations. Parenthesized numerals designate numbers of experiments upon which values are based

Molar conc. azide	Shumway stage	P_T	P_{AO}	P_I
0	10	13.0 ± 0.4 (3)	0.59 ± 0.09 (3)	0.12 ± 0.02 (3)
0	12–12^+	12.9 ± 0.7 (3)	0.59 ± 0.11 (3)	0.13 ± 0.02 (3)
10^{-5}	12^-	13.0 ± 0.3 (2)	0.65 (1)	0.16 ± 0.03 (2)
10^{-4}	$10\frac{1}{2}$	12.8 ± 0.5 (3)	0.43 ± 0.02 (2)	0.23 ± 0.02 (3)
10^{-3}	10^+	13.1 ± 0.4 (2)	0.44 ± 0.04 (2)	0.29 ± 0.05 (2)
10^{-2}	10	13.2 ± 0.5 (2)	0.44 ± 0.01 (2)	0.30 ± 0.01 (2)

Table II shows that the total phosphorus content of *R. pipiens* gastrulae, exposed for 24 hours to 10^{-5} to 10^{-2} *M* azide, is the same as that of untreated control gastrulae. This is an important point because (together with the data in Table I) it suggests that such embryos are practically undamaged, otherwise a certain amount of leakage of phosphorus might be expected. The other two phosphorus fractions, however, exhibit changes, for as the concentration of environmental azide is increased, there is an increase of the inorganic phosphorus content of treated embryos, accompanied by a quantitatively similar decrease in the level of acid-soluble organic phosphorus. Furthermore, as the concentration of environmental azide is progressively elevated, there is a parallel increase in the severity of gastrular retardation.

The data summarized in Table III show that a similar set of results may be obtained with 2, 4 dinitrophenol at concentrations of 10^{-5} *M*, or lower. At concentrations greater than 10^{-5} *M*, however, gastrulae are damaged to such an extent that they begin to leak phosphorus; this is clearly shown by a correlated decline in the levels of all three phosphorus fractions.

Finally, it should be noted that the gastrulation of untreated control embryos is unaccompanied by any significant alterations of phosphorus balance.

TABLE III

Developmental stage and phosphorus balance of R. pipiens gastrulae after 24 hours exposure to 2,4 dinitrophenol (DNP), 14–15° C. For meanings of column headings, see section on Methods. Numerals following ± designate standard deviations

Molar conc. DNP	Shumway stage	P_T	P_{AO}	P_I	No. expts.
0	10^-–10	13.1 ± 0.7	0.59 ± 0.07	0.13 ± 0.01	2
0	12	13.1 ± 0.1	0.58 ± 0.04	0.13 ± 0.01	2
10^{-6}	12	12.9 ± 0.1	0.60 ± 0.09	0.17 ± 0.05	2
10^{-5}	10^+–11^-	12.6 ± 0.2	0.45 ± 0.01	0.31 ± 0.02	2
5×10^{-5}	10^-	11.9	0.34	0.22	1
10^{-4}	10^-–10	12.4 ± 0.3	0.19 ± 0.09	0.08 ± 0.08	2

Those results suggest the following interpretation. If the movements of gastrulation of normal untreated embryos demand an available supply of phosphate-bond energy, the resulting draughts upon esterified phosphorus are immediately reimbursed, and the phosphorus balance is steadily maintained. This result is in full agreement with the data of Barth and Jaeger (1947), summarized in their Table 1. It is also consistent with the view that gastrulation is a complex of morphogenetic movements whose execution requires no expenditure of energy. The results with azide and dinitrophenol suggest the contrary, however, because of the correlation between the presence of these inhibitors, the reduction of esterified phosphorus, the elevation of inorganic phosphorus, and the retardation of gastrular movements. For it is difficult to explain this correlation except by assuming that in the presence of inhibitors the production of esterified phosphorus is uncoupled from its utilization as a source of morphogenetic energy because it is made available to enzymes catalyzing its remineralization (see the remarks at the beginning of this paper). Of the complex of movements, Gregg and Ornstein (1953) have presented evidence suggesting that epiboly is the most sensitive to treatment with dinitrophenol, while

TABLE IV

Developmental stage and phosphorus balance of R. pipiens gastrulae after 24 hours anaerobiosis, 14°–15° C. For meanings of column headings, see section on Methods. Numerals following ± designate standard deviations. P values in A and B are listed separately in order that the former may be compared with those obtained from hybrid embryos prepared from the same five clutches of eggs (Table V)

	Treatment	Shumway stage	P_T	P_{AO}	P_I	No. expts.
	Control	10	13.5 ± 0.4	0.58 ± 0.04	0.13 ± 0.02	5
A	Control	12	13.2 ± 1.0	0.55 ± 0.03	0.13 ± 0.01	5
	Nitrogen	11	13.5 ± 1.1	0.56 ± 0.05	0.15 ± 0.01	5
	Control	10^+	12.4	0.58	0.12	1
B	Control	12	13.00	0.52	0.13	1
	Nitrogen	11	12.9	0.52	0.18	1
	Control	10	12.4	0.58	0.13	1
C	Control	12^-	12.5	0.60	0.13	1
	Hydrogen	11^-	12.5	0.64	0.18	1

epiboly and notochordal elongation are most affected by the presence of azide; but the exact relations of their results to the present ones are yet to be worked out.

Anaerobiosis. Table IV summarizes the results of experiments designed to show the effects of 24 hours anaerobiosis on the phosphorus balance of *R. pipiens* gastrulae. Embryos are morphologically retarded under these conditions, but, in general, there is no alteration of the phosphorus balance other than a slight elevation of the inorganic phosphorus level. The total phosphorus and acid-soluble organic phosphorus levels are unaffected. There is thus a considerable morphogenetic effect of anaerobiosis, apparently unaccompanied by a decrease in the stored phosphate-bond energy potentially available. This conclusion is not in agreement with that of Barth and Jaeger, who found that anaerobioses of 10 to 22 hours duration resulted in a considerable decrease of ADP–ATP phosphorus. Their fractionation procedure is not strictly comparable with ours, however, and the apparent discrepancy cannot be resolved without further investigation.

TABLE V

Developmental stage and phosphorus balance of R. pipiens ♀ × R. sylvatica ♂ gastrulae after 24 hours anaerobiosis, 14–15° C. For meanings of column headings, see section on Methods. Numerals following ± designate standard deviations. These values should be compared with those in part A of Table IV, obtained from R. pipiens embryos prepared from the same five clutches of eggs

Treatment	Shumway stage*	P_T	P_{AO}	P_I	No. expts.
Control	10	13.2 ± 1.0	0.57 ± 0.04	0.12 ± 0.01	5
Control	12	13.7 ± 0.7	0.55 ± 0.05	0.12 ± 0.01	5
Nitrogen	11	13.6 ± 1.0	0.56 ± 0.05	0.14 ± 0.02	5

* The stages assigned are those of simultaneously developing *R. pipiens* control embryos (Table IV, A).

Embryos in the hybrid *R. pipiens* ♀ × *R. sylvatica* ♂ fail to gastrulate, but remain alive during the whole time required for control *R. pipiens* embryos to reach the hatching stage (Moore, 1946; see review by Gregg, 1957). They are characterized by low respiratory rates and by low rates of aerobic and anaerobic glycolysis. The expectation that they might therefore find it more difficult than normal embryos to maintain esterified phosphorus stores under the stress of anaerobiosis was confirmed by the data of Barth and Jaeger. Our own experiments do not bear out this expectation, for they show (Table V) that hybrid embryos under anaerobiosis do not alter their phosphorus balance to a greater extent than *R. pipiens* controls. But it is not clear that these results are in genuine disagreement with those of Barth and Jaeger, for the reason stated at the end of the preceding paragraph.

Summary

1. *Rana pipiens* gastrulae treated with non-damaging concentrations of sodium azide or 2, 4 dinitrophenol for 24 hours at 14–15° C. exhibit a reversible retardation of morphogenetic movements, a diminished store of acid-soluble organic phosphorus, an elevated content of inorganic phosphorus and an unaltered total phosphorus content.

2. Anaerobiosis for 24 hours at 14–15° C. does not alter the phosphorus balance of *R. pipiens* gastrulae, or of gastrula-arrested hybrids of *R. pipiens* ♀♀ with *R. pipiens* ♂♂, beyond a slight elevation of the inorganic phosphorus level.

3. These results are discussed briefly in respect to the energy-requirements of the morphogenetic movements of gastrulation.

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